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PRINCIPAL INVESTIGATOR: James Shull, Ph.D.

CONTRACTING ORGANIZATION: University of Nebraska Medical Center
Omaha, Nebraska 68198-6810

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James Shull, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Nebraska Medical Center
Omaha, Nebraska 68198 -6810

e-mail:
jshull@unmc.edu

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13. ABSTRACT ***(Maximum 200 Words)***

Our laboratory has played a leading role in establishing the female ACI rat as a physiologically relevant animal model for the study of breast cancer etiology. We were the first to demonstrate that the naturally occurring estrogen, 17β-estradiol (E2), induces rapid development of mammary cancers in the female ACI rat (1). We have demonstrated that the unique susceptibility of the ACI rat to E2-induced mammary cancers is inherited in ACI x Copenhagen (COP) and ACI x Brown Norway (BN) genetic crosses as a complex dominant trait (2), and most recently have mapped three genetic loci that confer and/or modulate susceptibility to E2-induced mammary cancers (3). In this USAMRMC funded research we are testing the hypothesis that genomic instability, specifically loss of heterozygosity (LOH), is an important contributing factor in the etiology of E2-induced mammary cancers. The primary experimental approach is to utilize a battery of polymorphic genetic loci to assess LOH in mammary tumors induced by E2 in ACI/COP F1 and ACI/BN F1 rats. To date, we have demonstrated that E2-induced mammary cancers exhibit genomic instability. Assays for detecting LOH have been established. Preliminary data indicate that LOH occurs within regions of the rat genome demonstrated by us to contain gene(s) that confer and/or modulate susceptibility to E2-induced mammary cancers.

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James D. Shull 10-25-99

PI - Signature Date

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Principal Investigator: James D. Shull, Ph.D.

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INTRODUCTION

Estrogens are inextricably implicated in the etiology of breast cancers in humans. Our laboratory has played a leading role in establishing the female ACI rat as a physiologically relevant animal model for the study of breast cancer etiology. We were the first to demonstrate that the naturally occurring estrogen, 17β-estradiol (E2), induces rapid development of mammary cancers in the female ACI rat (1). We have demonstrated that the unique susceptibility of the ACI rat to E2-induced mammary cancers is inherited in ACI x Copenhagen (COP) and ACI x Brown Norway (BN) genetic crosses as a complex dominant trait (2), and most recently have mapped three genetic loci that confer and/or modulate susceptibility to E2-induced mammary cancers (3). In this USAMRMC funded research we are testing the hypothesis that genomic instability, specifically loss of heterozygosity (LOH), is an important contributing factor in the etiology of E2-induced mammary cancers. The primary experimental approach is to utilize a battery of polymorphic genetic loci to assess LOH in mammary tumors induced by E2 in ACI/COP F1 and ACI/BN F1 rats. To date, we have demonstrated that E2-induced mammary cancers exhibit genomic instability. Assays for detecting LOH have been established. Preliminary data indicate that LOH occurs within regions of the rat genome demonstrated by us to contain gene(s) that confer and/or modulate susceptibility to E2-induced mammary cancers.

BODY

This progress report summarizes work completed between May 15, 1999 and October 15, 1999. Initiation of this research was delayed as the Specific Aims and Statement of Work were revised to eliminate perceived overlap with an award from the National Cancer Institute. Progress relating to each of the revised Statement of Work Tasks is summarized herein.

Task 1. Each mammary tumor will be evaluated histologically to define its type as comedo or papillary carcinoma and to assess its extent of invasiveness. Year 1 and beyond as additional tumors become available.

This task has been accomplished for tumors obtained to date. All E2-induced mammary tumors were adenocarcinoma of the comedo, cribriform or papillary types, with the comedo form being the predominant cancer type. Invasive features were observed in a significant fraction of these mammary tumors. Additional E2-induced mammary tumors will be generated over the next 18 months in work funded by NIH grant CA77876.

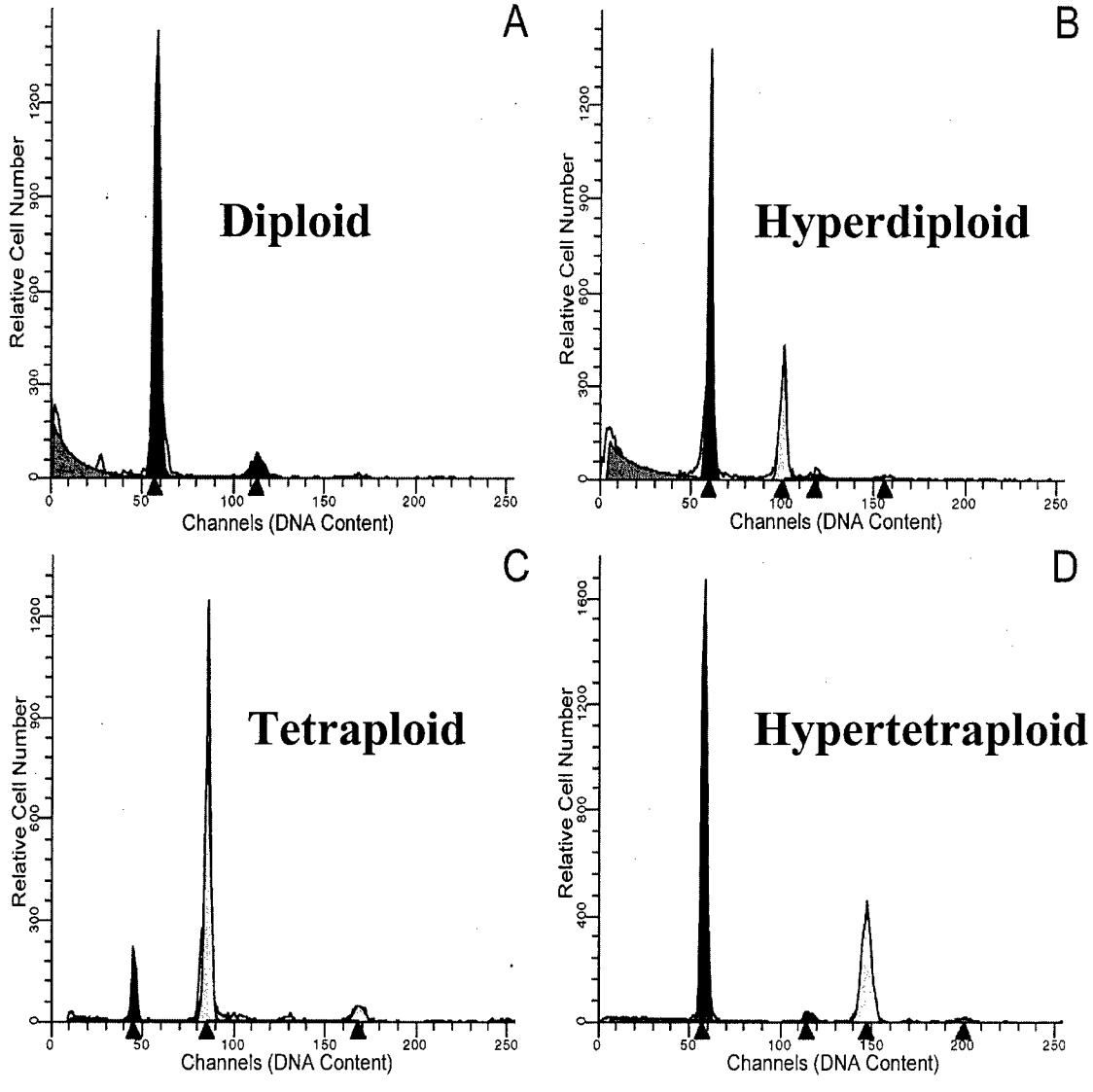
Task 2. Cells will be isolated from each mammary tumor and analyzed by flow cytometry to quantify cellular DNA content and assess ploidy. DNA content different from 2N and 4N will be considered to be an indicator of aneuploidy. Year 1 and beyond as additional tumors become available.

Methods: Frozen mammary tumors and spleens from the tumor bearing animals were thawed on ice, cut into small pieces using a razor blade and mechanically dissociated in PBS using a Stomacher laboratory blender (Tekmar, Cincinnati, OH). The dissociated cells were pelleted by centrifugation, resuspended in RPMI culture media (Life Technologies, Gaithersburg, MD), filtered through Spectra/Mesh polymer macrofiltration filters (Spectrum, Laguna Hills, CA) and counted. Following dilution to approximately 1 million cells per ml in Vendelov's reagent the cells were analyzed using a FACStar Plus flow cytometer (Becton Dickinson, San Jose, CA). Cells prepared from normal spleen were used as an internal ploidy standard. The resulting data were analyzed using ModFit DNA analysis software (Becton Dickinson).

Results and Discussion: Flow cytometric analysis of DNA content within cells isolated from mammary cancers induced in female ACI rats by E2 indicated that the majority of these tumors exhibit genomic instability (Figure 1). Within a group of fifteen E2-induced mammary cancers, five (33%) of the tumors exhibited a normal DNA profile, where the majority of cells displayed a diploid DNA content (Figure 1A). Seven (47%) tumors contained a significant fraction of cells which exhibited a hyperdiploid DNA content (Figure 1B), two (13%) tumors contained a large fraction of cells that were tetraploid in their DNA content (Figure 1C), and one (7%) of the 15 mammary tumors contained a large fraction of cells that were hypertetraploid in their DNA content (Figure 1D). These

data clearly illustrate that different E2-induced mammary carcinomas are heterogeneous with respect to ploidy, with approximately 66% of the examined tumors exhibiting clear features of aneuploidy. This observation is noteworthy as the mammary tumors that develop in rats and mice treated with dimethylbenz[*a*]anthracene have been reported to exhibit a diploid DNA profile.

Figure 1. Mammary Tumors Induced in Female ACI Rats by Chronic Treatment with 17β-estradiol Exhibit Aneuploidy. Cells were isolated from mammary tumors induced by E2. DNA content was quantified by flow cytometry.



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Task 3. A panel of appropriately located simple sequence repeat (SSR) loci will be identified that are polymorphic between the ACI and COP and the ACI and BN strains from among those mapped in the rat genome.

This task is being accomplished on a chromosome by chromosome basis and will be continued throughout the remainder of the research project. Data generated in the analysis of chromosomes 5 and 18 are summarized below.

Task 4. DNA will be isolated from each mammary tumor as well as from the spleen of each mammary tumor bearing F1 animal. Each of the polymorphic SSR loci identified in #3 will be amplified using polymerase chain reaction (PCR) and commercially available primers. The amplified DNAs will be subjected to electrophoresis on denaturing acrylamide gels and the amount of DNA product generated from the ACI and COP or ACI and BN alleles at each locus will be quantified using a phosphorimager. LOH will be revealed as a strain specific loss of an SSR locus in the mammary tumor relative to the spleen from the same animal. Suspected LOH will be confirmed by repeating the analyses at flanking loci. Years 1 through 3 as warranted by new releases of the rat genome map.

Methods: The source of rat tissues used in this project were from experiments supported by NIH grant CA77876. In the NIH funded experiments, mammary tumors were induced in ACI/COP F1 and ACI/BN F1 female rats by treatment with E2, administered from subcutaneous Silastic implants as described by us previously (1). No funds from the USAMRMC were used for purchase or housing of live animals.

Methods for evaluating LOH were established and optimized. Genomic DNA was isolated from 10 mg of rat spleen and 25 mg of rat mammary tumor using QIAamp tissue kits (QIAGEN). Each DNA (30 ng) was amplified by PCR using Platinum Taq DNA Polymerase (Gibco) and standard methods as described in our original application; [32P]dCTP (Amersham) was included in each PCR reaction, resulting in generation of labeled PCR products. Oligodeoxynucleotide primers for amplifying the different polymorphic SSR loci on each of the rat chromosomes were obtained from Research Genetics, Inc. The PCR products were separated on 8% denaturing polyacrylamide sequencing gels. Each PCR reaction was assayed in triplicate. Following drying, the gels were visualized by autoradiography on a Phosphorimager (Molecular Dynamics), and the intensities of the DNA bands generated from the ACI and COP alleles were quantified using ImageQuant software (Molecular Dynamics). The average (± standard deviation, n = 3) pixel intensity generated from the ACI and COP alleles from spleen and mammary tumor DNA was determined for each of several SSR loci. An LOH index for each polymorphic locus was calculated by: 1) dividing the intensity of the ACI product by the intensity of the COP product generated from each mammary tumor and spleen DNA; 2) comparing the ACI/COP ratio from the mammary tumor with the ACI/COP ratio from the spleen using Student's t test; and, 3) subsequently dividing the ACI/COP ratio from each mammary tumor by the ACI/COP ratio from each spleen as illustrated in Formula 1.

Formula 1. $(ACI_{tumor} / COP_{tumor}) / (ACI_{spleen} / COP_{spleen}) = LOH\ index$

The standard deviation of each ACI/COP ratio (n =3) rarely exceeded 10% of the mean. An LOH index that was equal to or greater than 1.25 was interpreted to suggest loss of the COP allele within the genome of the mammary tumor at the locus assayed. An LOH index that was equal to or less than 0.75 was interpreted to suggest loss of the ACI allele within the genome of the mammary tumor at the locus assayed.

Results and Discussion: In a project funded by NIH grant R01 CA77876, we have mapped three loci that confer and/or modulate susceptibility to E2-induced mammary cancers. *Emca1*, located on rat chromosome 5, was mapped using F2 progeny from ACI x COP and ACI x BN intercrosses. *Emca2*, located on rat chromosome 18, and *Emca3*, located on rat chromosome 2, were revealed only in the ACI x BN intercross. Consequently, we are focusing our attention initially on these chromosomes.

Ten polymorphic loci identified on rat chromosome 5 were evaluated for possible LOH in a panel of 21 mammary tumors induced by E2 in ACI/COP F1 progeny. Strong evidence of LOH was observed in four of the 21 E2-induced mammary cancers. LOH indices greater than 1.25 were observed in tumor 47 at seven of the 10 loci; in tumor 114 at eight of the 10 loci; and in tumor 163 at six of 10 loci. These data suggest that a large segment, perhaps all, of the COP copy of rat chromosome 5 has been lost at some point during development of these three tumors. Interestingly, tumor 116 exhibited an LOH index less than 0.75 at nine of the 10 loci examined. This latter observation is consistent with loss of the ACI copy of rat chromosome 5 during development of tumor 116. Three additional tumors exhibited loss of the COP allele at two of the ten loci, and two tumors exhibited loss of the COP allele at one of the ten loci. Two tumors exhibited loss of the ACI allele at one of the ten loci. Together, these data indicate that genomic instability involving rat chromosome 5 is associated with approximately 50% of the mammary tumors induced in ACI/COP F1 animals by E2. At this time, a cause and effect relationship between LOH and mammary tumor development cannot be concluded.

Six polymorphic loci identified on rat chromosome 18 have been evaluated at the present time. Tumor 464 exhibited loss of the COP allele at four of the six loci; tumors 47 and 114 exhibited loss of the COP allele at three of the six loci; and tumors 112, 115, and 146 exhibited loss of the COP allele at two of the six loci. Twelve additional tumors exhibited loss of the COP allele at one of the six polymorphic loci evaluated on chromosome 18. Together, these data indicate that loss of COP alleles for loci on chromosome 18 are common in mammary tumors induced by E2, being observed in a total of 18 of the 21 tumors evaluated thus far.

Task 5. The resulting data will be analyzed to determine: 1) the extent of LOH occurring across the rat genome; 2) whether the observed LOH occurs selectively in specific regions of the genome; and 3) whether LOH within a specific region of the genome correlates with tumor type or invasiveness. Years 2 and 3.

Data analysis is ongoing as data are collected.

Task 6. The data will be communicated to the scientific community through presentation at appropriate meetings and publication in peer reviewed journals. Years 2 through 3.

Select data from these ongoing studies were communicated by Dr. Shull in an invited presentation at the Gordon Conference on Hormonal Carcinogenesis held in August of 1999. These data will also be briefly summarized at an upcoming meeting of rat genetics and genomics to be held at the Cold Spring Harbor Laboratory in December of 1999. A manuscript presenting the data summarized in Figure 1 will be submitted in the next few weeks.

Task 7. All required progress reports and communications will be prepared and submitted to the Army Breast Cancer Research Program. Years 1 through 3.

In progress and to be continued as necessary.

KEY RESEARCH ACCOMPLISHMENTS

1. Flow cytometric analyses of E2-induced mammary tumors have been performed indicating that a large fraction of these tumors exhibit aneuploidy.

2. Methods for evaluating LOH have been established and optimized.

3. Appropriate polymorphic loci have been identified for evaluating LOH in mammary tumors induced in ACI/COP F1 rats by chronic treatment with E2.

4. Rat chromosome 5, harboring *Emca1*, has been screened for LOH. Resulting data suggest large segments of chromosome 5 are lost during development of E2-induced mammary cancers.

5. Rat chromosome 18, harboring *Emca2*, has been screened for LOH. Resulting data indicate that loss of COP alleles are common during development of E2-induced mammary cancers.

REPORTABLE OUTCOMES

Manuscripts, abstracts, presentations: A manuscript is in preparation for fall 1999 submission. Two oral presentations at international meetings have or will be presented by the end of 1999.

Patents: A patent application on Emca1, Emca2 and Emca3, identified with funding from the NIH has been submitted. The research funded by the USAMRMC will enhance our understanding of the roles of the genes residing within these loci in the development of E2-induced mammary cancers.

Degrees: None.

Development of cell lines: None.

Informatics such as databases and animal models: None.

Funding applied for: None at present. Additional applications anticipated.

Employment or research opportunities: None.

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CONCLUSIONS

Data generated to date suggest that genomic instability and LOH are common events during E2-induced mammary tumorigenesis. It is anticipated that this information will enhance our understanding of the molecular events leading to mammary cancer development. Our research on estrogen-induced mammary tumorigenesis is illustrating for the first time the physiological relevance of the ACI rat as an animal model for the study of breast cancer etiology.

No changes in the research plan are anticipated at this time.

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